Electronic Supplementary Information (ESI)

Alleviation of symptoms in Alzheimer's disease by diminishing A*β*-neurotoxicity and neuroinflammation

Tao Yang,‡[a] Zhenzhu Zhu,‡[a] Enmao Yin,[a] Yanqing Wang,[b] Changli Zhang,[c] Hao Yuan,[d] Hongmei Zhang,[a] Suxing Jin,[a] Zijian Guo,[d] and Xiaoyong Wang*[a]

[a] *State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 210023, P. R. China. E-mail: boxwxy@nju.edu.cn*

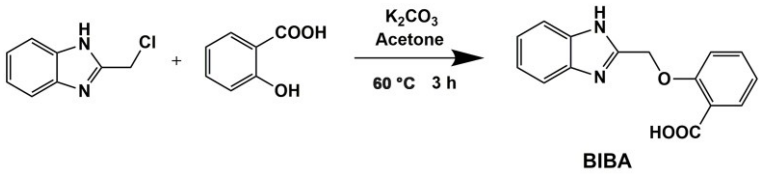
[b] *School of Chemistry and Chemical Engineering, Yancheng Teachers University, Yancheng 224002, P. R. China.*

[c] *Department of Chemistry, Nanjing Xiaozhuang College, Nanjing, 210017, P. R. China.*

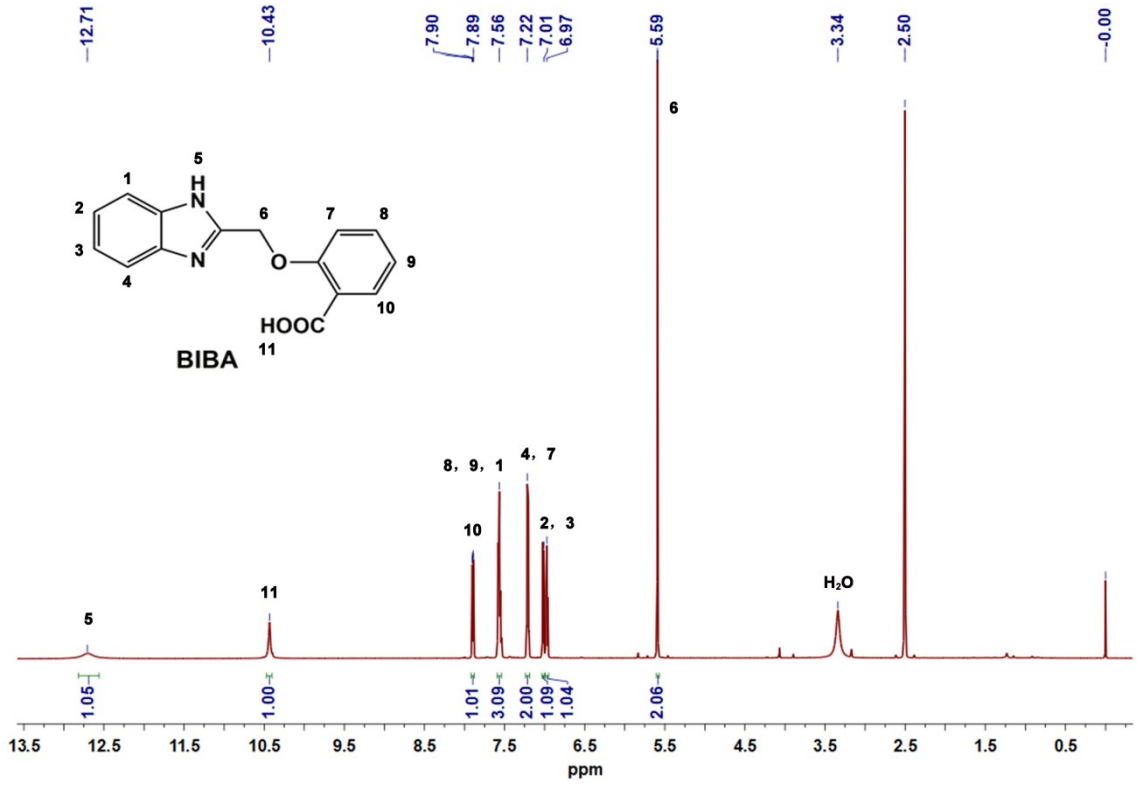
[d] *State Key Laboratory of Coordination Chemistry, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210023, P. R. China*

‡*These authors contributed equally.*

Supplementary Scheme, Figures and Tables.



Scheme S1 Synthetic route to BIBA.



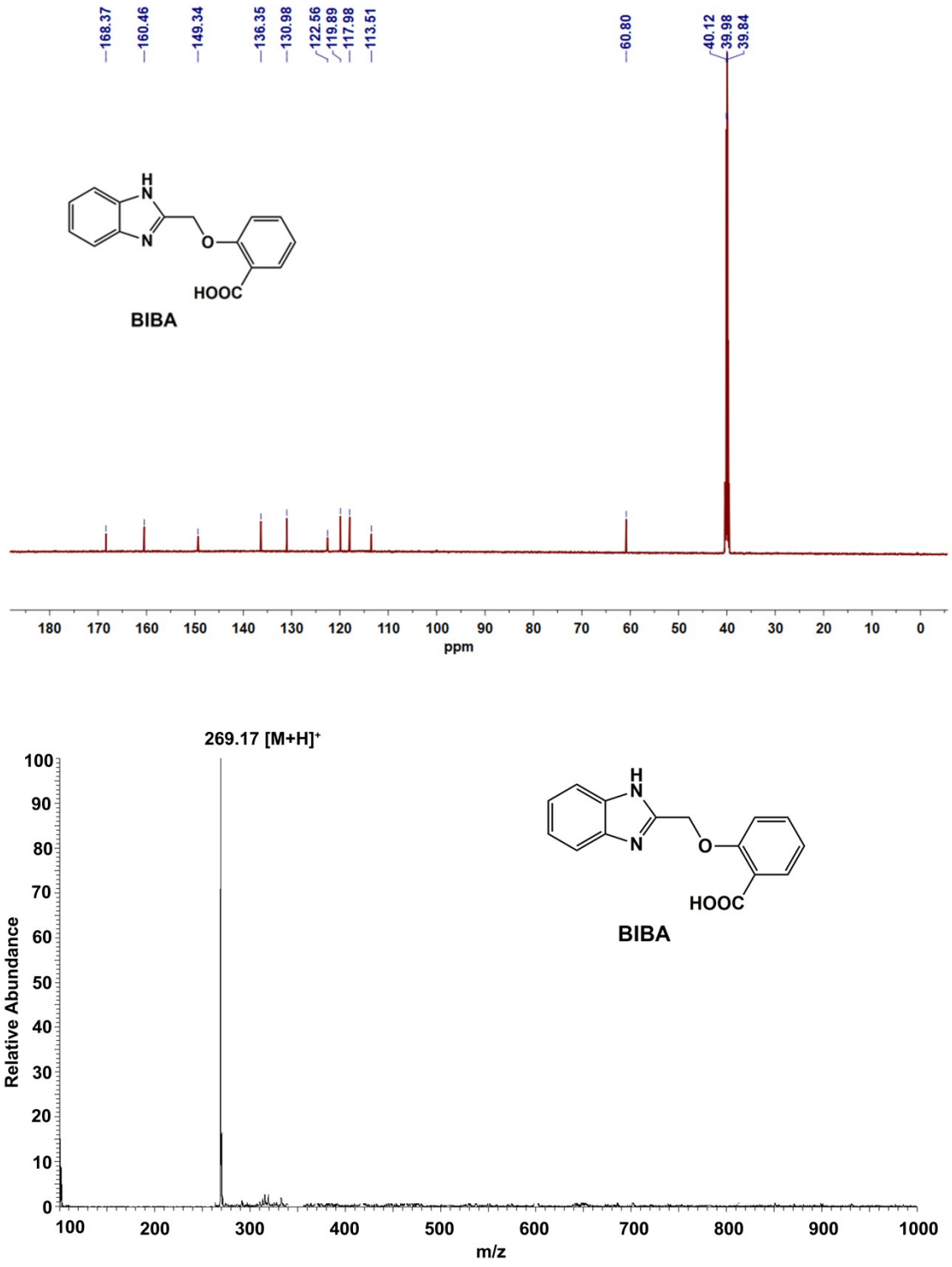


Fig. S1 ^{1}H-, ^{13}C-NMR (DMSO-d_6), and ESI-MS spectra for BIBA.

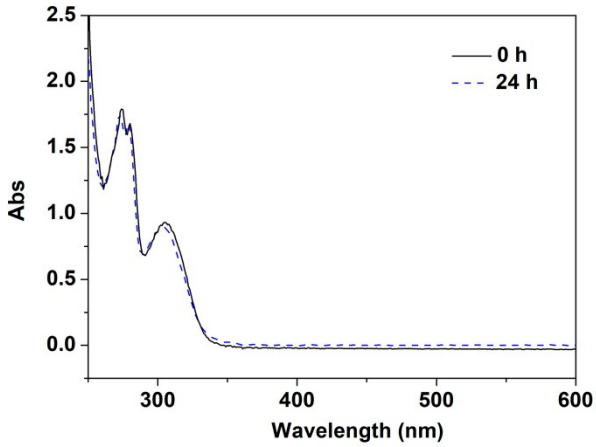


Fig. S2 UV-Vis absorption spectra of BIBA (100 μM) in buffer (5 mM Tris-HCl, 50 mM NaCl, pH 7.4) after incubation at 37 ℃ for 0 and 24 h respectively.

Table S1 Molecular weight (MW), ClogP, hydrogen bond acceptor (HBA), hydrogen bond donor (HBD), polar surface area (PSA), and logBB values of BIBA.[a]

	MW	ClogP	HBA	HBD	PSA	logBBb
BIBA	268.08	2.784	4	2	70.92	–0.487
Lipinski's rules	≤ 450	≤ 5	≤ 10	≤ 5	≤ 90 Å^2	

[a]ClogP and PSA of BIBA was predicted by ChemOffice. [b]logBB = –0.0148 × PSA + 0.152 × ClogP + 0.139; compounds with logBB > 0.3 are able to cross the BBB readily, with logBB <–1.0 are only poorly distributed to the brain.

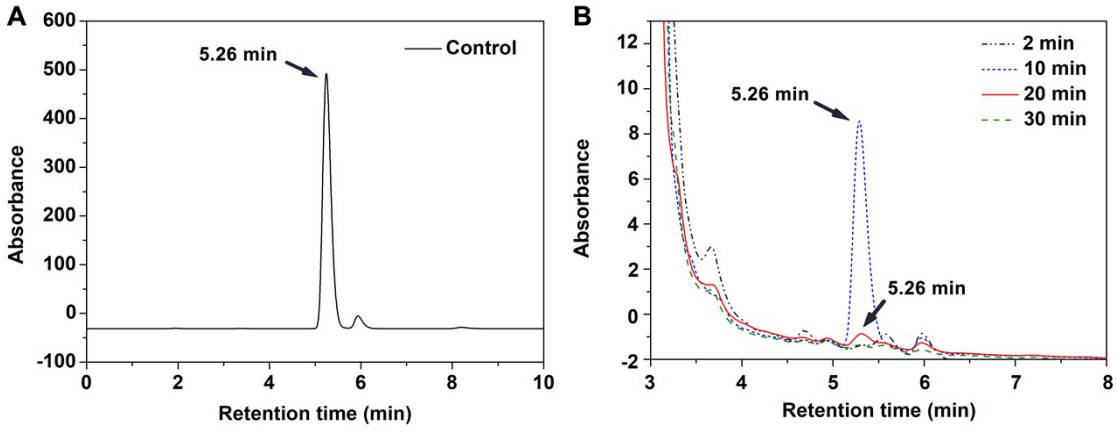


Fig. S3 HPLC spectra of BIBA (A) and BIBA from the brain extraction of C57BL/6J mice (B) at different time (2, 10, 20 and 30 min) after injection via the tail vein (20 mg Kg^{-1}).

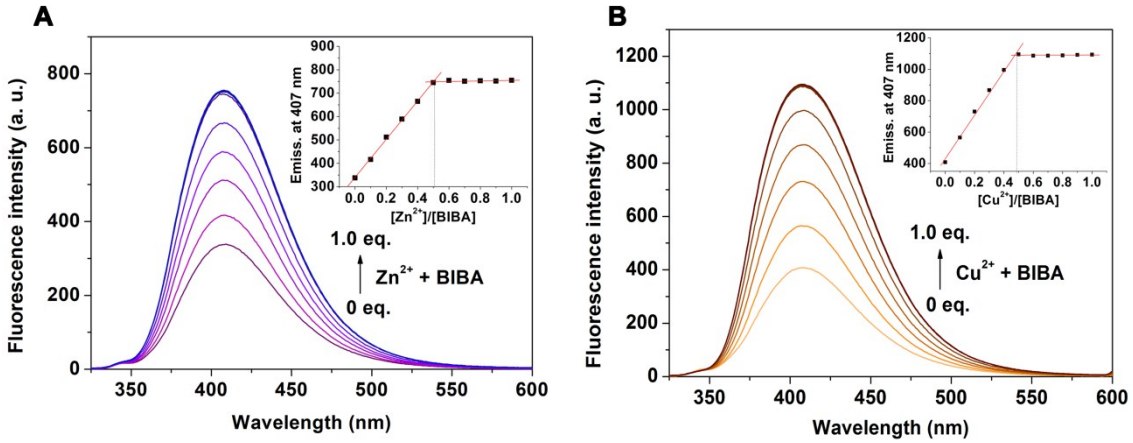


Fig. S4 Fluorescence spectra of BIBA (20 μM, λ_{ex} = 308 nm) upon addition of increasing concentrations of Zn^{2+} (A) or Cu^{2+} (B) in the buffer (20 mM Tris-HCl, 150 mM NaCl, 4‰ v/v DMSO, pH 7.4). Inset shows the emission intensity of BIBA at 407 nm versus different [Zn^{2+} or Cu^{2+}]/[BIBA] ratio.

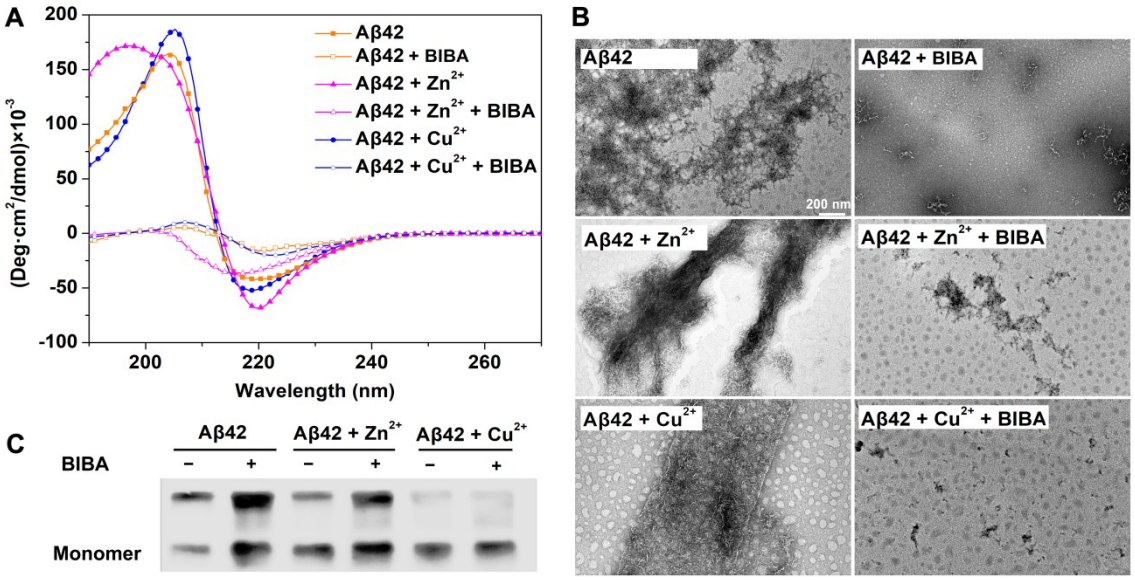


Fig. S5 Prevention of self or metal-induced Aβ42 aggregation by BIBA. (A) CD spectra, (B) TEM, (C) western blot of Aβ42 without or with metal ions and BIBA ([Aβ42] = [Zn^{2+}/Cu^{2+}] = 10 μM, [BIBA] = 20 μM).

Table S2 Assignment of the peaks (m/z) observed in the ESI-MS spectra for the interaction of BIBA with Aβ40 and Zn^{2+} (see Fig. 2D).

Species	Calculated m/z	Observed m/z
$[BIBA + H]^+$	269.27	269.08
$[BIBA + HAC + H_2O + H]^+$	333.32	332.92
$[BIBA + CH_3OH + HAC + H_2O + Na]^+$	387.14	386.58
$[BIBA + Zn + 2Cl + CH_3OH + H]^+$	437.64	437.42
$[A\beta40 + 3BIBA + Zn + H_2O + 3H]^{5+}$	1044.23	1044.50
$[A\beta40 + 2Zn + 4Cl + HCOOH + 4H]^{4+}$	1163.14	1163.50
$[A\beta40 + 3BIBA + Zn + 2Cl + H_2O + 4H]^{4+}$	1323.26	1322.92
$[A\beta40 + 4BIBA + 3Zn + 4Cl + H_2O + 2H]^{4+}$	1440.25	1440.75

Table S3 Assignment of the peaks (m/z) observed in the ESI-MS spectra for the interaction of BIBA with Aβ40 and Cu^{2+} (see Fig. 2E).

Species	Calculated m/z	Observed m/z
$[BIBA + H]^+$	269.27	269.33
$[BIBA + H_2O + Na]^+$	309.27	308.83
$[BIBA + HAC + H_2O + H]^+$	333.32	332.83
$[BIBA + CH_3OH + HAC + H_2O + Na]^+$	387.34	386.75
$[2BIBA + Cu + 2Cl + H_2O + K]^+$	728.13	727.75
$[A\beta40 + Cu + 2Cl + CH_3OH + 4H]^{4+}$	1125.10	1125.75
$[A\beta40 + 4BIBA + 3Cu + 2Cl + 4H_2O + 2HAC]^{4+}$	1457.16	1456.83
$[A\beta40 + 5BIBA + 2Cu + HAC + CH_3OH + 4H_2O]^{4+}$	1487.11	1486.75
$[A\beta40 + 2BIBA + 2Cu + 2Cl + 2HAC + CH_3OH + H_2O + H]^{3+}$	1735.85	1736.00

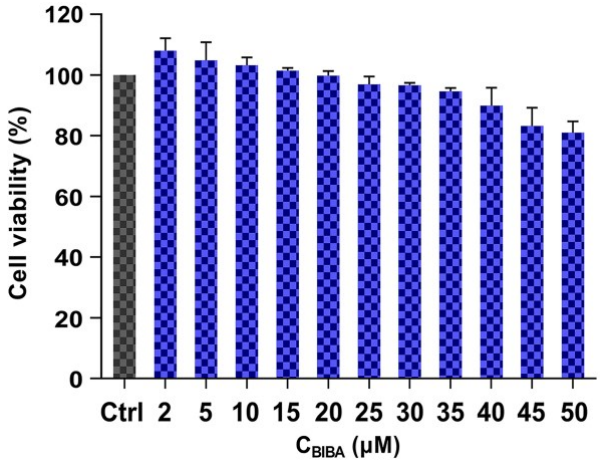


Fig. S6 Viability of N2a cells after treatment with different concentrations of BIBA for 24 h determined by the MTT assay.

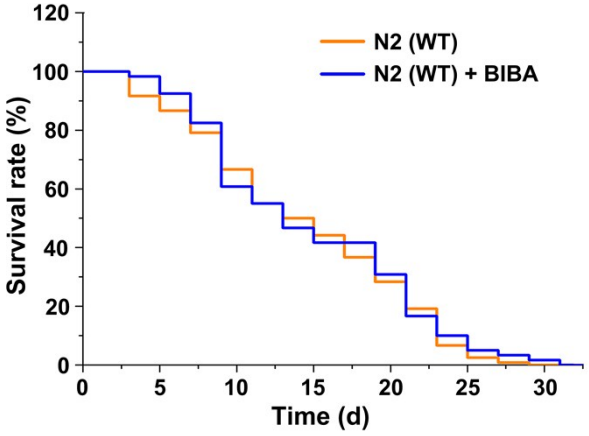


Fig. S7 Effects of BIBA on the lifespan of N2 worms.

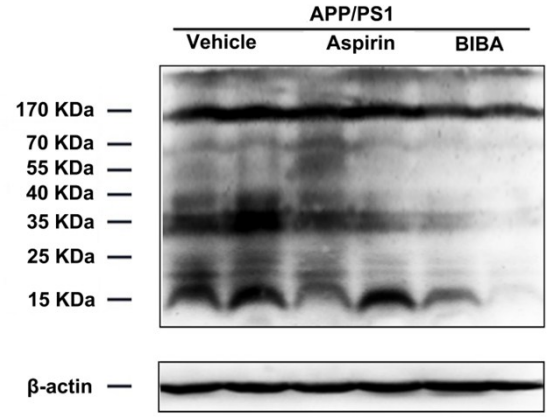


Fig. S8 Western blot of Aβ species in the brain of APP/PS1 mice after treatment with vehicle, aspirin or BIBA. The mice were treated with BIBA (2 mg Kg^{-1}) and aspirin (1.4 mg Kg^{-1}), respectively, every 3 days for 3 months.

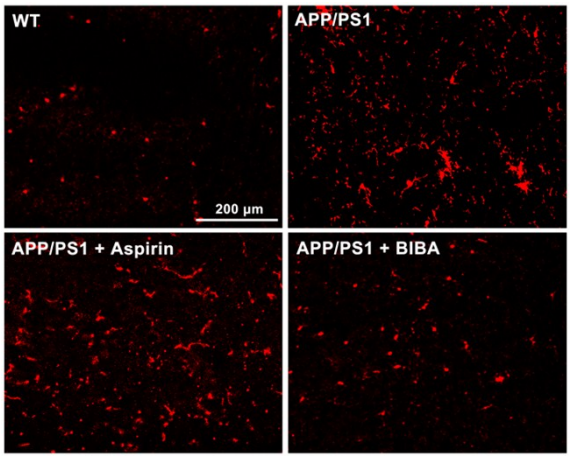


Fig. S9 Reduction of microglia overactivation. Histochemical analyses of Iba-1 (red) in the brain of WT and APP/PS1 mice after treatment with vehicle, aspirin (1.4 mg Kg^{-1}) and BIBA (2 mg Kg^{-1}), respectively, every 3 days for 3 months.

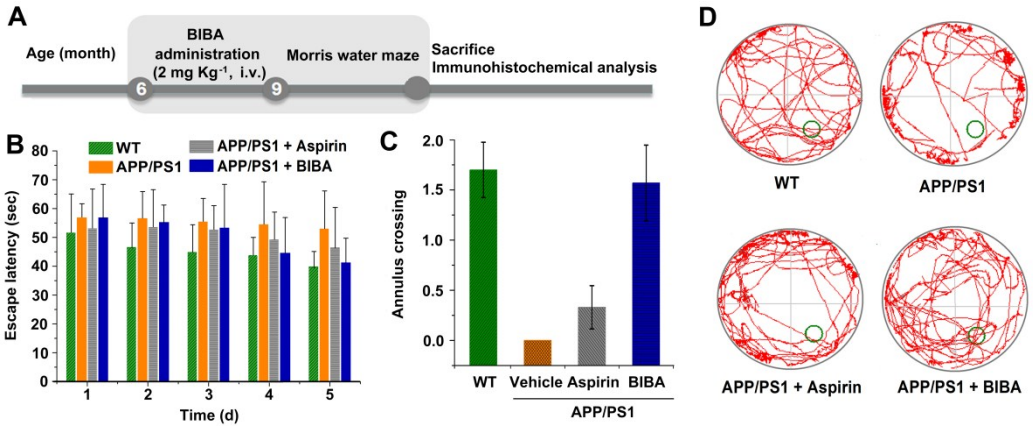


Fig. S10 Rescue of cognitive deficits in (6-month-old, male) APP/PS1 mice. (A) Time course of behavioral tests and immunohistochemical analysis. Mice (n = 6) were treated with BIBA (2 mg Kg^{-1}) and aspirin (1.4 mg Kg^{-1}), respectively, every 3 days for 3 months; (B) escape latency during platform trials in the Morris water maze; (C) number of annulus crossing in probe test; and (D) representative tracing graphs of open field test.

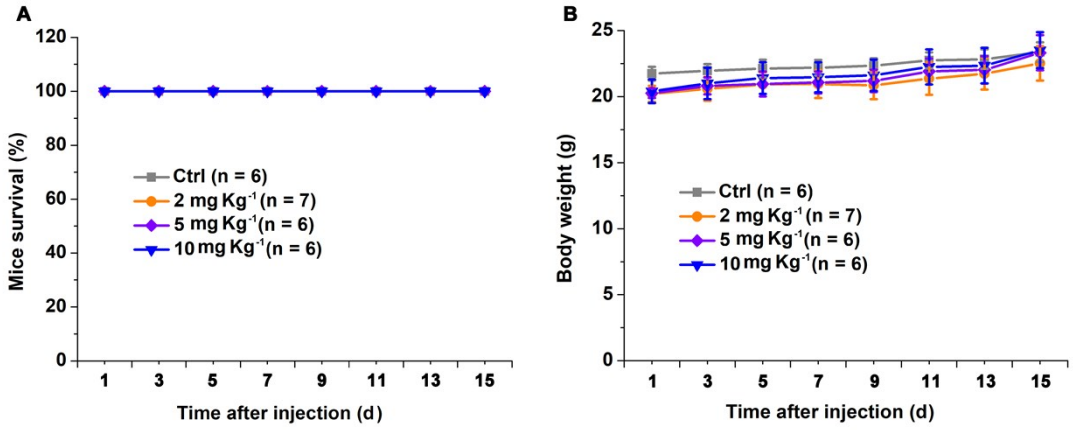


Fig. S11 Survival rate (A) and body weight (B) of C57BL/6 mice after injection of BIBA via the tail vein every other day at a dosage of 0 (n = 6), 2 (n = 7), 5 (n = 6) or 10 mg Kg^{-1} (n = 6), respectively.

Table S4 Interaction energies between BIBA and responsive amino acid residues in molecular docking.

Aβ40 monomer (PDB 1BA4)		Aβ40 fibrils (PDB 2LMO)	
amino acid	interaction energy (KJ mol^{-1})	amino acid residues	interaction energy (KJ mol^{-1})
Arg-5	–6.9645	Ala-30(A)	–2.3986
His-6	–18.5083	Ile-31(A)	–5.5805
Gln-15	–20.419	Ile-32(A)	–8.8720
Lys-16	–4.1642	Ala-30(B)	–9.4560
Val-18	–8.5251	Ile-31(B)	–8.7679
Phe-19	–21.0038	Ile-32(B)	–15.2147
Glu-22	–4.6692	Ala-21(C)	–3.2784
		Val-24(C)	–12.8290
		Gly-25(C)	–8.2679
		Ala-30(C)	–8.9467
		Ile-31(C)	–6.6073
		Ile-32(C)	–6.8784
		Phe-20(D)	–5.6857

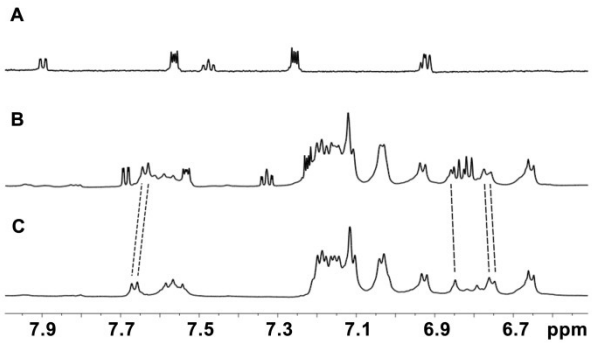


Fig. S12 ^{1}H NMR spectra of BIBA (A) and Aβ40 (0.2 mM) in the presence (B) and absence (C) of BIBA (0.4 mM) after incubation at 37 °C and pH 7.4 for 24 h in the mixture containing 82% H_2O, 10% D_2O and 8% DMSO-d_6.

Experimental section

Materials and animals

Reagents used in this study were all of analytical grade, purchased from commercial suppliers and used as received unless otherwise stated. 2-(Chloromethyl) benzimidazole and salicylic acid were purchased from Macklin Inc. (Shanghai, China). Zinc chloride, copper chloride and thioflavine S (ThS) were purchased from Sigma-Aldrich. Human Aβ40 and Aβ42 was purchased from GL Biochem Ltd. (Shanghai, China). Stock solution of Aβ40 and Aβ42 was prepared according to the literature method.[1] BIBA and aspirin stock solutions (5 mM) were obtained by dissolving each compound in dimethyl sulfoxide (DMSO) and filtered using a 0.22 μm filter (organic system). All solutions and buffers were prepared using Milli-Q water, and filtered through a 0.22 μm filter (Millipore) before use. Dulbecco's Modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Keygen Biotech and Hyclone, respectively. Cell-lysis RIPA buffer and BCA protein assay kit were purchased from Beyotime Biotechnology (Shanghai, China). The ECL reagent (6883), GAPDH (ab181602), bax (ab32503), bcl-2 (ab182858), bad (ab32445), cytochrome-c (ab76237), caspase-3 (ab184787), TNF-α (ab215188), Iba-1 (ab178847), Goat anti-rabbit IgG H&L (Alexa Fluor® 647) (ab150079), Goat anti-rat IgG H&L (Alexa Fluor® 647) (ab150159), Goat anti-rabbit HRP (ab97051) antibodies were purchased from Abcam. Anti-Aβ monoclonal antibody (Catalog SIG-39320) was purchased from Covance. Secondary HRP-conjugated goat anti-mouse anibody (A0216) were purchased from Beyotime Biotechnology (Shanghai, China). CD86 (B7-2) monoclonal antibody (GL1) was purchased from ThermoFisher scientific.

Mouse N2a neuroblastoma cells (ATCC) and BV-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 ℃ and 5 % CO_2.

C. elegans strains, including transgenic strains CL4176 (*dvIs27 [myo-3p::Aβ (1-42)::let-851 3′*UTR + rol-6(su1006)]) that express Aβ gene in muscle tissue and its control strain CL802 (*smg-1 (cc546ts)I; rol-6 (su1006)*II) that does not express Aβ gene were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN, USA). The worms were cultured on the standard Nematode Growth Media (NGM) plates spotted with *E.coli* strain OP50 grown in Luria Broth (LB) medium. NGM (12.0 g agar, 1.5 g peptone, 1.8 g NaCl, 590 mL H_2O) was autoclaved, and $CaCl_2$ (0.6 mL, 1 M), cholesterol (0.6 mL, 5 mg mL^{-1} in ethanol), $MgSO_4$ (0.6 mL, 1 M), KPO_4 buffer (15 mL, 1 M; 17.8 g KH_2PO_4, 54.2 g K_2HPO_4, 500 mL, pH 6.0) were added to NGM. An aliquot (5.0 mL) of NGM was transferred into each petri dish (Φ 35 mm) and allowed to solidify overnight. LB medium was prepared by dissolving NaCl (10.0 g), yeast extract (5.0 g), and tryptone (10.0 g) in double distilled water (1.0 L) and autoclaved. *E.coli* strain OP50 was grown in LB medium by shaking overnight. Each plate containing solidified NGM was spotted with 100 μL of OP50 and allowed to dry for 6 h, it was then cultured in 37 ℃ overnight, cooled at room tempeture and stored in 4 ℃ for later use.

C57BL/6J mice and APPswe/PSEN1 transgenic mice were purchased from the Model Animal Research Center of Nanjing University (MARC) and Guangdong Medical Laboratory Animal Center (GDMLAC), respectively. All mice were housed in a laboratory animal breeding room under controlled temperature with an alternating 12 h light-dark cycle and access to food and water *ad libitum*. All animal experiments were performed in accord with institutional animal use and care regulations approved by MARC and GDMLAC. All mice were humanely killed with an overdose of anesthetics and perfused transcardially with saline, and the brains were harvested immediately after completion of medication and behavioral test.

Methods

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-600 spectrometer. Electrospray ionization mass spectra (ESI-MS) were obtained on an LCQ Fleet electrospray mass spectrometer. Circular dichroism (CD) spectra were determined by using a JASCO J-810 automatic recording spectropolarimeter (Tokyo, Japan). Fluorescence spectra were recorded on a HORIBA Fluoromax-4P fluorescence spectrometer. Transmission electron microscopic (TEM) images were obtained on a transmission electron microscope (JEOL, JEM-2100 LaB6). Data in this work were expressed as means ± standard deviation (S. D.). Two-way Analysis of Variance (ANOVA) was used to ascertain significant differences between the control and drug-treated groups by the GraphPad Primer 7.00 software. Differences at the $p \leq 0.05$ level were considered as significant.

Synthesis of BIBA

It was synthesized according to the literature approach.[2] The anhydrous acetone solution (40 mL) of salicylic acid (0.228 g, 1.65 mmol) and potassium carbonate (0.415 g, 3.0 mmol) were heated to reflux under N_2 for 15 min. Acetone solution of 2-(chloromethyl)benzimidazole (0.439 g, 1.65 mmol) was added and stirred at 60 ℃ overnight. The precipitate was filtered and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography and dried to obtain the final product (0.199 g, yield: 45%). ^{1}H NMR (DMSO-d_6, 600 MHz, δ, ppm): 12.71 (s, 1H, benzimidazole), 10.43 (s, 1H, -COOH), 7.22–7.20 (m, 2H, benzimidazole), 7.58–7.56 (m, 2H, benzimidazole), 7.90–7.89 (d, 1H, Ph), 7.56–7.54 (m, 1H, Ph), 7.02–7.01 (d, 1H, Ph), 6.99–6.96 (t, 1H, Ph), 5.59 (s, 2H, -CH_2-), 3.34 (s, H_2O). ^{13}C NMR (DMSO-d_6, 100 MHz, δ, ppm): 168.37, 160.46, 149.34, 136.35, 130.98, 122.56, 117.98, 113.51, 60.80. ESI-MS (positive mode, CH_3OH, m/z) found (calcd) for $C_{15}H_{12}N_2O_3$ (m/z): 269.17 (269.08) $[M + H]^+$.

Metal binding

The fluorescence titrations of BIBA (λ_{ex} = 308 nm) were carried out by adding aliquots of $ZnCl_2$ or $CuCl_2$ aqueous solution (5 mM, 1.2 μL) to 3 mL of BIBA solution (20 μM, 20 mM Tris-HCl, 150 mM NaCl, 4‰ v/v DMSO, pH 7.4) in a cuvette.

Stability

BIBA was dissolved in buffer (5 mM Tris-HCl, 50 mM NaCl, pH 7.4) at 100 μM containing 2% DMSO and incubated at 37 ℃ for 0 and 24 h. UV-vis absorption spectrum of the complex was determined on a Specord 200 UV-visible spectrophotometer.

Octanol/water partition coefficient (Log $P_{o/w}$)

The lipophilicity of BIBA was measured in an octanol/buffer system using the shake-flask method and UV spectroscopy. Solutions of BIBA (50, 75, 100 μM) containing DMSO (1%) were prepared in the PBS (pH 7.4) presaturated with octanol. Equal volumes (2.0 mL) of the solution and octanol presaturated with PBS were mixed and shaken at room temperature for 24 h and then separated into two phases by centrifugation. The concentration of the solute in the aqueous phase was determined by spectrophotometry (λ_{max} = 295 nm). According to the law of mass conservation, the drug concentration of the corresponding octanol phase and the lipo−hydro partition coefficient $P_{o/w}$ ($P_{o/w} = C_o/C_w = A_o/A_w$, where A stands for absorbance) were calculated. The log *P* values were calculated on the average of three independent measurements.

Inhibition of metal-free or metal-induced A*β* aggregation

Each sample solution (199.2 μL, 20 mM Tris-HCl/150 mM NaCl, pH 7.4) consisting of A*β*40 or A*β*42, Zn^{2+} or Cu^{2+} (10 μM), and DMSO (4‰) was incubated at 37 ℃ for 15 min. BIBA (0.8 μL, 5 mM) was added and incubated at 37 ℃ for 24 h. The degree of A*β* aggregation was studied by TEM, CD spectroscopy and western blotting. The sample solutions for CD spectroscopy were prepared by the same way as described above and incubated at 37 ℃ for 12 h. CD spectra were recorded on a JASCO J-810 automatic recording spectropolarimeter (Tokyo, Japan) in the range of 190–300 nm. The samples for ^{1}H NMR spectra were prepared by adding BIBA (0.4 mM) to the solution of A*β*40 (0.2 mM) in 10% D_2O, 82% H_2O and 8% DMSO-d_6, and incubating at 37 °C for 24 h. NMR spectra were recorded with water suppression.

Cytotoxicity

The cytotoxicity of BIBA was tested by the MTT assay on N2a cells *via* the cleavage of MTT to purple formazan cystals by mitochondrial dehydrogenases. Briefly, N2a cells were seeded in a 96-well flat bottomed microplate at 6000 cells/well and incubated at 37 ℃ in a humidified atmosphere with 5% CO_2. A*β*42, Zn^{2+} or Cu^{2+} (10 μM) was added into the tissue at 37 ℃ for 15 min, followed by addition of BIBA (20 μM, final DMSO concentration 4‰). After incubation at 37 ℃ for 24 h, MTT (50 μL, 5 mg/mL) was added to each sample solution. The samples were re-incubated for 4 h at 37 ℃ in a humidified atmosphere with 5% CO_2. The supernatants were removed and the formazan crystals were dissolved in 300 μL DMSO. The absorbance at 490 nm was determined using a Varioskan Flash microplate reader.

Protein expression in N2a or BV-2 cells

N2a or BV-2 cells (3 × 10^6 cells/well) were planted into a 10 cm plate (10% foetal bovine serum in DMEM). Stock solution of BIBA (20 μM, 4‰ DMSO) was added to the cells 1 h before treatment with LPS (1 μg mL^{-1}) or A*β*42 (10 μM), and the cells were incubated at 37 ℃ for 12 h. The cells were collected and lysed on ice in a cell-lysis RIPA buffer and centrifuged at 12,000g and 4 ℃ for 15 min. The supernatants were collected and assayed for protein concentration by the BCA protein assay kit. The supernatants were dissolved in loading buffer and boiled at 95 ℃ for 15 min. Each sample was separated by SDS polyacrylamide gel electrophoresis (PAGE), followed by transferring it onto polyvinylidene difluoride (PVDF) membranes. The membranes were taken out and blocked for 1 h at room temperature with 5% non-fat milk powder. The membranes were incubated overnight with various primary antibody (diluted by PBS-T buffer) at 4 ℃, and then were taken out, rinsed with PBS-T buffer for 4 × 5 min and incubated with the HRP-conjugated goat anti-rabit antibody (1: 10000) for 1 h at room temperature. Bands were visualized with SuperSignal (Millipore). The membranes were then rechecked with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as internal reference to ensure equal protein loading. The mean densities of protein reactive bands were analyzed by Image J (National Institutes of Health, USA).

Immunocytochemistry

After various treatment described above, BV-2 cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.5% Triton for 15 min. The cells were washed by cold PBS for 5 min, blocked for 15 min at room temperature with 0.5% BSA being dissolved in PBS. The cells were incubated with the primary antibody (CD86, 1:200, Thermo Scientific) overnight at 4 ℃. After incubation, BV-2 cells were washed twice with PBS and incubated with specific secondary

antibody for 1 h at room temperature.

Paralysis assay

Synchronized *C. elegans* strain CL4176 worms expressing muscle-specific A*β*42 were transferred onto NGM plates containing *E. coli* OP50 mixed with either BIBA or aspirin, using strain CL802 as the control. The worms were allowed to lay eggs at 15 °C for 2 h, and then picked off the adult worms and allowed the eggs to hatch and grow at 15 ℃ for about 36 h. The temperature was upshifted to 25 °C when worms were at the third larval stage (L3). Scoring the paralysis of worms after initiation of upshift to 25 °C for 24 h. At this point, all worms in the population should have reached the fourth larval (L4) stage. The number of paralyzed worms treated with drug was counted every 2 h until all worms were paralyzed.

Lifespan assay

The *C. elegans* strain CL4176 was cultured for 2–3 generations before using for lifespan analysis in N2 at 16 ℃.[3] For each assay, synchronous L4 larvae or young adults were transferred to each NGM plate containing *E. coli* OP50 in the presence or absence of BIBA or aspirin (20 μM), treated with 40 μM of FUdR to inhibit the growth of progeny and scored every other day until all worms on the plates died.

Staining of A*β* plaques in *C. elegans*

Age-synchronized transgenic CL4176 worms fed with vehicle, aspirin or BIBA were incubated at 25 ℃ for 48 h. The worms were stained with 0.125% ThS in 50% ethanol for 2 min. The samples were then destained by transferring to fresh NGM plates for 2 h, mounted on slides and observed with a microscope (DM4000B; Leica, Germany) equipped with a digital camera. Fluorescence images were acquired using a CDD.

Western blot analysis of A*β* species and cytokines in *C. elegans*

After the treatments as described in paralysis assay, the worms were collected, washed with M9 buffer, flash frozen in liquid nitrogen, sonicated in M9 buffer, and heated with sample buffer containing loading buffer at 95 ℃ for 10 min. The proteins were cooled and equal amounts of the total protein (60–70 mg) were loaded in each lane. A*β* species were detected with 6E10 monoclonal antibody (1:1000, Covance); Secondary HRP-conjugated goat anti-mouse antibody (1:5000). *β*-Actin was detected with anti-*β*-actin antibody (1:2000, Abcam). The inflammatory cytokines were detected as described above.

Toxicity

C57BL/6J mice (8-week-old, male) were randomized and divided into three experimental groups and one control group (6~7 mice in each). Experimental mice received 2, 5, and 10 mg Kg^{-1} of BIBA via tail vein, while the control mice received DMSO (5% in normal saline) every other day for two weeks. During administration of BIBA, body weight of mice was recorded every other day, changes in mortality and hair loss were observed.

BBB penetration assay

C57BL/6J mice (8-week-old, female) were used for studying BBB penetration capability of BIBA. Briefly, each mouse was given BIBA (20 mg Kg^{-1}, dissolved in a 20 : 80 DMSO/H_2O mixture, 150 μL) via the tail vein injection. The mice were sacrificed after 2, 10, 20, 30 min, and the brain tissue was collected and rinsed with cold PBS. Brain tissue was Lysed with NP40 Lysis Buffer (600 μL) and homogenized. The homogenate was mixed with MeOH (1400 μL) overnight at 4 ℃ and centrifuged. The supernatants were collected and concentrated. The samples were further mixed with MeOH (300 μL) and centrifuged. The supernatants were collected, and the sample (20 μL) was subjected to HPLC analysis using a C18 reverse-phase column (250 mm × 4.6 mm) (Agilent) and a MeOH/H_2O mixture (80 : 20, v/v) as the mobile phase. The flow rate was 1 mL min^{-1}. The absorption wavelength was 308 nm.

ThS staining and immunostaining of brain slices

After behavioural tests, the brains of the wild-type and APP/PS1 mice were removed and fixed in 4% paraformaldehyde (pH 7.4). The fixed brain samples were then immersed in 30% sucrose for cryoprotection and cut into 30-μm-thick slices using a Cryosta (Microm HM 525, Thermo Scientific, Waltham, MA, USA). The sliced brains were stained with 500 μM of ThS for 7 min. The sections were then rinsed with PBS. For immunostaining, the slides were stained with anti-Iba1 antibody (1:200 dilution). Alexa Fluor® 647-conjugated secondary antibodies were used for fluorescence detection. Fluorescent observation was performed using an LSM-710 (Zeiss, Germany).

Immunoblot analysis of brain lysates

After behavioural tests, the brains of wild-type and APP/PS1 mice were collected and homogenized in RIPA buffer (20

mM Tris–HCl, pH 7.5, 50 mM NaCl, 0.5% NP-40, 4 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate and protease inhibitor cocktail) on ice for 15 min and centrifuged at 16,000 g and 4°C for 30 min. The supernatant of brain lysates was dissolved in loading buffer and boiled at 95 °C for 15 min, which was then used for western blot as described above.

Behavioural studies

BIBA (2 mg Kg^{-1}) or aspirin (1.4 mg Kg^{-1}) was injected into the tail vein of mice every three days. After drugs treatment, these mice were subjected to the Morris water maze (mwm) test to examine the learning and spatial memory abilities according to the reported procedures.[4] A white circular platform (diameter 8 cm; height 30 cm) was submerged 1–2 cm beneath the water surface, food-grade titanium dioxide was added to ensure the platform invisible for the mice, and the water tempreture was maintained at 22 ± 1 ℃. The temperature of the pool and the location of the platform (15 cm from the south-west (sw) wall) were maintained constant from day 1 to 5. On each side of the walls of the four quadrants, distinct colored paper were placed as a visual positional hint. A video tracking camera was mounted onto the ceiling directly above the center of the pool to monitor the subject-swimming parameters. The mice were trained for 5 consecutive days with 4 trials per day as acquisition trials, assigned 4 pseudorandom starting points (e, se, nw and n). A total of 20 trials were performed with each mouse in the acquisition phase within 5 days. During each trial, the mouse was freely allowed to find the submerged escape platform in opaque water of a circular pool and the latency to reach the platform was measured. The animals were positioned facing the wall at one of the 4 starting positions and allowed to locate the submerged platform. A successful trial was recorded when a mouse found the platform within 60 s. The mouse was allowed to stay on the platform for 5 s prior to being returned to its home cage dry for 20 min. If a mouse was unable to locate the platform within 60 s, it was gently guided to the platform and allowed to stay on it for 5 s. The time required (latency) to find the escape platform and the swimming speed were measured. On the sixth day, probe test was performed to determine memory retention without the platform for 60 sec. Each mouse was placed into the opposite quadrant of the target zone where the platform was removed (time in the target zone). Numbers of annulus crossing in probe were also calculated. Data were recorded and analyzed using a video camera-based ethovision system (nodulus, the netherlands).

Docking of BIBA with Aβ40

The molecular docking program Autodock 4.2.3 and Autodock tools 1.5.4 were employed to dock BIBA to Aβ40.[5] The geometry of BIBA was optimized using density functional theory (DFT) at the level of B3LYP/6-311G by using the program Gaussian 09.[6] The molecular structures of Aβ40 monomer (PDB: 1BA4) and fibrils (PDB: 2LMO) were obtained from Protein Data Bank.[7,8] All water molecules and ligands were removed from the structure of Aβ40, and essential hydrogen atoms, Kollman united atom type charges, and salvation parameters were added in the protein model. The Gasteiger partial charges were added to atoms of BIBA, and rotatable bonds were defined. In addition, the grid box sizes of BIBA–Aβ40 were 126 Å × 126 Å × 126 Å, with grid spacing of 0.375 Å, while the GA population size was set to 150, the maximum number of energy evaluation was set to 25×10^6, the number of GA runs was 100, and others used were default parameters. The lowest energy conformations were selected and figures of BIBA docked with Aβ40 monomers or fibrils were depicted using Pymol.

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